SPEC

Attorney ~~Docket No.: 16761/110~~

~~Inventors:~~ ~~Roy A. Black~~
~~Raymond James Paxton~~
~~Wolfram Bode~~
~~Klaus Maskos~~
~~Carlos Fernandez-Catalan~~
~~James Ming Chen~~
~~Jeremy Ian Levin~~

~~TITLE OF THE INVENTION~~

~~Crystalline TNF-α-Converting Enzyme and Uses Thereof~~

Replace the paragraph on page 1, starting at line 5 with the following

~~INFORMATION ON RELATED APPLICATIONS~~

[This application claims the priority benefit of U.S. provisional patent application serial No. 60/073,709, filed February 4, 1998, U.S. patent application serial No. 09/050,083, filed March 30, 1998 (which will be converted to a US provisional application pursuant to a petition filed on January 27, 1999), and US provisional patent application titled "Crystalline TNF-α-Converting Enzyme and Uses Thereof," filed January 27, 1999.] This application is a continuation of U.S. Serial No. 09/611,722 filed July 6, 2000, which is a divisional of U.S. Serial No. 09/244,984 filed on February 4, 1999, which claims the priority benefit of U.S. Provisional Application No. 60/117,476 filed on January 27, 1999, U.S. Provisional Application No. 60/135,499 filed on March 30, 1998, and U.S. Provisional Application No. 60/073,709 filed on February 4, 1998. The contents of these prior applications are hereby incorporated by reference.

~~BACKGROUND OF THE INVENTION~~

~~The cytokine tumor necrosis factor-α (TNFα) plays a role in the induction of inflammatory reactions and is known to be cytotoxic towards tumor cells. TNFα, however, also may cause severe damage to the human body when produced~~ in

SPEC

~~Attorney Docket No.: 16761/170~~

Replace paragraph on page 3, Starting @ line 3 as follows

~~**SUMMARY OF THE INVENTION**~~

According to one aspect of the invention, there is provided a composition comprising a polypeptide in crystalline form, wherein the polypeptide is a TNF-α-converting enzyme polypeptide. In one embodiment, the TNF-α-converting enzyme polypeptide comprises the TNF-α-converting enzyme catalytic domain. In another embodiment, the TNF-α-converting enzyme polypeptide is the expression product of a polynucleotide encoding the pro and catalytic domains of TNF-α-converting enzyme. In a further embodiment, the TNF-α-converting enzyme polypeptide is the expression product of a polynucleotide encoding the amino acid residues 1-477 of TNF-α-converting enzyme. In yet another embodiment, the polynucleotide is substituted such that amino acid residue Ser266 is changed to Ala and amino acid residue Asn542 is changed to Gln, and wherein a second polynucleotide encoding the sequence Gly-Ser-$(His)_6$ (**SEQ ID NO: 2**) is fused to the C-terminus.

According to another aspect of the ~~invention~~, the compositions ~~above~~ further comprising a binding partner suitable for co-crystallization with the TNF-α-converting enzyme polypeptide. In one embodiment, the binding partner is a hydroxamate-based binding partner. In another embodiment, the binding partner is N-{D,L-2-(hydroxyaminocarbonyl)methyl-4-methylpentanoyl}-L-3-amino-2-dimethylbutanoyl-L-alanine,2-(amino)ethyl amide.

According to further embodiments, the compositions above have a crystal structure diffracting to 2.0 Å, are monoclinic, have a unit cell comprising ~~four~~ crystallographically independent TNF-α-converting enzyme catalytic domain (TCD) molecules, have the TCD molecules are in an asymmetric unit, and/or have monoclinic space group $P2_1$ and the cell has the constants a=61.38 Å, b=126.27 Å, c=81.27 Å, and β=107.41°.

In still another embodiment of the invention, the polypeptides above ~~are~~ characterized by the structure coordinates according to Table 1, ~~or~~ a substantial part thereof.

SPEC

Replace paragraph on page 7, line 19-
page 9, line 4 as follows

storage regions containing information for visually depicting a characteristic of one of a plurality of amino acids; wherein said second-type storage regions are logically associated with said first-type storage regions in said video memory to represent a geometric arrangement of at least one characteristic of said at least a portion of said TNF-α converting enzyme polypeptide in said three dimensional space. In one embodiment, the second-type storage regions are logically associated with said first-type storage regions in said video memory to represent a geometric arrangement of at least one characteristic of a catalytic domain portion of said TNF-α converting enzyme polypeptide in said three dimensional space. In another embodiment, the first-type storage regions and said second-type storage regions are regions of a semiconductor memory. In yet another embodiment, the first-type storage regions and said second-type storage regions are regions of an optical disk. In still another embodiment, the first-type storage regions and said second-type storage regions are regions of a magnetic memory. In a further embodiment, the video memory comprises a plurality of first-type and second-type storage regions.

In a still further aspect of the invention, there is provided a method of identifying a compound that associates with TNF-α-converting enzyme, comprising (A) designing an associating compound for said polypeptide that forms a bond with the TNF-α-converting enzyme catalytic domain based on x-ray diffraction coordinates of a TNF-α-converting enzyme polypeptide crystal; (B) synthesizing said compound; and (C) determining the associate capability of said compound with said TNF-α-converting enzyme. In one embodiment, the associating compound is an inhibitor, mediator, or other compound that regulates TNF-α-converting enzyme activity. In another embodiment, the associating compound is a competitive inhibitor, un-competitive inhibitor, or non-competitive inhibitor. In still another embodiment, the coordinates are the coordinates of Table 1, or a substantial part thereof. In a further embodiment, the TNF-α-converting enzyme polypeptide crystal comprises the TNF-α-converting enzyme catalytic domain. In still another

embodiment, the TNF-α-converting enzyme polypeptide is the expression product of a polynucleotide encoding the pro and catalytic domains of TNF-α-converting enzyme. In yet another embodiment, the TNF-α-converting enzyme polypeptide is the expression product of a polynucleotide encoding the amino acid residues 1-477 of TNF-α-converting enzyme. In another embodiment, the polynucleotide is substituted such that amino acid residue Ser266 is changed to Ala and amino acid residue Asn542 is changed to Gln, and wherein a second polynucleotide encoding the sequence Gly-Ser-$(His)_6$ (**SEQ ID NO: 2**) is fused to the C-terminus. In a further embodiment, the TNF-α-converting enzyme polypeptide crystal is co-crystallized with a binding partner. In still another embodiment, the binding partner is a hydroxamate-based binding partner or N-{D,L-2-(hydroxyaminocarbonyl) methyl-4-methylpentanoyl}-L-3-amino-2-dimethylbutanoyl-L-alanine,2-(amino)ethyl amide. In yet other embodiments, the TNF-α-converting enzyme polypeptide crystal has a crystal structure diffracting to 2.0 Å, is monoclinic, has a unit cell comprising four crystallographically independent TNF-α-converting enzyme catalytic domain (TCD) molecules, has the TCD molecules are in an asymmetric unit, and/or is of monoclinic space group $P2_1$ and the cell has the constants a=61.38 Å, b=126.27 Å, c=81.27 Å, and β=107.41°. In still another embodiment, the invention the associating compound is designed to associate with the S1' region of TNF-α-converting enzyme. In yet another embodiment, the associating compound is designed to associate with the S1'S3' pocket of TNF-α-converting enzyme. In still other embodiments of the invention, the associating compound is designed to (i) incorporate a moiety that chelates zinc, (ii) form a hydrogen bond with Leu348 or Gly349 of TNF-α-converting enzyme, (iii) introduce a non-polar group which occupies the S1' pocket of TNF-α-converting enzyme, (iv) introduce a group which lies within the channel joining S1' - S3' pockets of TNF-α-converting enzyme and which makes appropriate van der Waal contact with the channel, and/or (v) form a

SPEC

hydrogen bond with Leu348 or Gly349 on the backbone amide groups of TNF-α-converting enzyme.

~~These and other aspects of the invention will become apparent to the skilled artisan in view of the teachings contained herein.~~

SPEC

Replace paragraph on page 10, line 3 with the following

~~Attorney Docket No.: 16761/170~~

BRIEF DESCRIPTION OF THE FIGURES

Fig. 1: Figure 1 is a ribbon diagram of the TACE catalytic domain (TCD). The chain starts on the lower left back side, runs through the structural elements sI, hAI, hA, sII, hB, hB2, sIII, IV, IVa, sIVb, sV, hC, Met-turn and hD, and ends in the upper left back. The three disulfides are shown as connections, with the sulphurs given as small spheres. The catalytic zinc (central sphere) is liganded by the three imidazoles of His4O5, His4O9 and His415, and by the hydroxyl and the carbonyl oxygen atoms of the inhibitor hydroxamic acid group. The inhibitor mimicking interaction of primed-site residues of a peptide substrate is shown in full. Figure 1 was made using SETOR. See Evans, S. "SETOR: Hardware Lighted Three-Dimensional Solid Model Representations of Macromolecules" *J. Mol. Graph.* 11:134-138 (1993).

Figs. 2a and 2b: Figures 2a and 2b are solid surface representations of the catalytic domains of TACE (TCD) (Figure 2a) and MMP-3 (Figure 2b). The electrostatic surface potential is contoured from -15 (intense red) to 15 (intense blue) k_BT/e. Both active-site clefts run from left to right, with the catalytic zinc atoms (spheres) in the centers. In TACE, the bound inhibitor is shown in full structure, binding with its isobutyl (P1') and its Ala (P3') sidechains into the deep S1' and the novel S3' pockets. The orientation is similar to Fig. 1. Figures 2a and 2b were made using GRASP. Nicolls, A., Bharadwaj, R. and Houig, B., "Grasp - Graphical representation and analysis of surface properties," *Biophys.* 64, A166 (1993).

Fig. 3: Figure 3 aligns the catalytic domain sequences of adamalysin II (ADAM_CROAD) (**SEQ ID NO: 4**), TACE (**SEQ ID NO: 5**) and human ADAM 10 (hADAM1O) (**SEQ ID NO: 6**), according to their topological equivalence and sequence similarity, respectively. The residue numbers are due to the generic TACE numbering. Arrows and braces represent β-strands and α-helices in TACE.

Replace paragraph on page 12, line 7 as follows

~~Attorney Docket No.: 16761/1 16~~

The TACE amino acid sequence, or any part or residue thereof, ~~can be~~ found in Black *et al.*, "A Metalloproteinase disintigrin that releases tumour-necrosis factor-α from cells," *Nature* 385: 729-733 (Feb. 1997), herein incorporated in the entirety by reference. Variations in the amino acid sequence of TACE are within the present invention as well. All references to the TACE amino acid sequence contained herein refer to the sequence in Black *et al.*, supra.

As used herein, the TACE catalytic domain (TCD) refers to the portion of a TACE polypeptide between residues 215 and 477 and including the preceding furin cleavage site (residues 211-214), or any part thereof that is capable of cleaving the peptide PLAQAVRSSS (**SEQ ID NO: 1**).

Expression, Isolation and Purification of TACE Polypeptides

Tumor necrosis factor-α converting enzyme (TACE) is described in the published PCT application No. WO 96/41624. The application describes isolated nucleic acids encoding TACE or portions of TACE, expression vectors comprising a cDNA encoding TACE or portions thereof, and host cells transformed or transfected with the expression vectors comprising a cDNA encoding TACE or portions of TACE. The application further describes processes for producing TACE and portions thereof, for example by culturing transfected cells engineered to express TACE, followed by purification of the recombinantly produced TACE or portion thereof. Methods of isolating, expressing, and purifying a TACE polypeptide are described in detail in published PCT application No. WO 96/41624. The entirety of PCT 96/41624 is incorporated herein by reference.

According to the invention, cDNA encoding the signal peptide, pro and catalytic domains of TACE, *i.e.*, amino acid residues 1-477 is inserted into a suitable expression vector and expressed in a suitable cell line. The cDNA also may include other regions that facilitate expression or achieve other objects that

SPEC

SPEC

Replace paragraph on page 13, line 13 as follows

otherwise that do not depart from the essence of the invention, such as flanking regions.

The cDNAs encoding the TACE polypeptide, or functional portions thereof, such as the TCD, may be altered by addition, substitution, deletion, or insertion. Such alterations may be made, for example, to prevent glycosolation, prevent formation of incorrect or undesired disulfide bridges, and/or enhance expression. Examples of such alterations are described in WO 96/41624 and can be carried out by the methods described therein and other conventional methods. TACE may also be conjugated. Such conjugates may comprise peptides added to facilitate purification and/or identification. Such peptides include, for example, poly-His peptides. Conjugation is described in U.S. Patent No. 5,011,912 and Hopp *et al.*, *Bio/Technology* 6:1204 (1988).

In one embodiment of the invention, the cDNA encodes a TNF-α converting enzyme polypeptide comprising the signal peptide, pro and catalytic domains of TACE (TCD), residues 1-477, with Ser266 changed to Ala and Asn452 changed to Gln. These substitutions are useful in preventing N-linked glycosolation. Additionally, the sequence Gly-Ser(His)$_6$ **(SEQ ID NO: 2)** may be added to the C-terminus. The addition of the sequence Gly-Ser(His)$_6$ **(SEQ ID NO: 2)** facilitates purification of the polypeptide using metal-chelate affinity resins, such as Ni-NTA resins.

Recombinant expression vectors containing the nucleotide sequence encoding TACE, or a portion thereof, may be prepared using well known methods. Suitable host cells for expression of TACE polypeptides include prokaryotic, yeast, and higher eukaryotic cells. Vectors and host cells suitable for use in the present invention are described in WO 96/41624. Further examples of suitable expression systems that can be employed to express recombinant TACE according to the present invention include mammalian or insect host cell culture expression systems, including baculovirus systems in insect cells (See Luckow and Summers,

SPEC

Replace paragraph on page 14, line 16 as follows

Bio/Technology 6:47 (1988)) and mammalian cell lines such as COS-7 cells (Gluzman et al., *Cell* 23:175 (1981)). Additional examples are known in the art and include those described in WO 96/41624. In one embodiment of the invention, the TACE polypeptide is expressed in CHO cells. In this embodiment, the cells secrete a mixture of TACE polypeptide beginning with Val212 and Arg215.

In one embodiment, stable expressing cells may be selected by culturing the cells in a drug that kills those cells that do not incorporate the vector. Examples of suitable selection methods are described in, for example, Kaufman, R.J., "Selection and coamplification of heterologous genes in mammalian cells," *Methods in Enzymology*, 185:537-566 (1990).

Purification of the expressed TACE polypeptide may be carried out by any suitable means, such as those described in WO 96/41624. According to one aspect of the invention, it is preferable to obtain a TACE polypeptide that is suitable for crystallization. In obtaining a TACE polypeptide suitable for crystallization, it is important that the process for purifying the TACE polypeptide is sufficient to yield a polypeptide pure enough to properly crystallize.

A preferred method of purification starts with a suitable amount of medium from the culture of TACE-secreting cells. This medium is generally a supernate of the culture. The medium contains the TACE polypeptide to be purified. Preferably, the TACE polypeptide is recombinantly produced using DNA coding for the TACE polypeptide with the sequence altered to encode a conjugate or conjugates that facilitate purification. For example, the sequence encoding Gly-Ser-$(His)_6$ (**<u>SEQ ID NO: 2)</u>** may be added to the C-terminus to facilitate purification using metal-chelate resins.

The medium is concentrated, for example, by diafiltration. Suitable diafiltration units include a Millipore 10K cut-off, 1 ft^2 TFF diafiltration unit. A suitable buffer solution is then added to the concentrated medium. Any suitable

Replace paragraph on page 17, line 13 as follows

SPEC

with a binding partner described above. Exemplary means for obtaining the TACE polypeptide, as well as purification of the polypeptide are described above.

Crystals may be grown or formed by any suitable method, including drop vapor diffusion, batch, liquid bridge, and dialysis, and under any suitable conditions. Crystallization by drop vapor diffusion is often preferable. In addition, those of skill in the art will appreciate that the crystallization conditions may be varied. Various methods of crystallizing polypeptides are generally known in the art. See, for example, WO 95/35367, WO 97/15588, EP 646 599 A2, GB 2 306 961 A, and WO 97/08300.

In one embodiment of the invention, a DNA construct comprising TACE residues 1-477, with Ser266 changed to Ala, Asn452 changed to Gln, and the sequence Gly-Ser-$(His)_6$ **(SEQ ID NO: 2)** added to the C-terminus, may be expressed in CHO cells. These cells primarily secrete a processed mixture of TACE, about half beginning with Val212 and about half with Arg215. The mixture is purified as described above. The purified TACE polypeptide, with the added binding partner, is stored in a buffer as described above.

The TACE polypeptide and binding partner are co-crystallized. The TACE/binding partner solution, at a polypeptide concentration of about 5 mg/mL to about 12 mg/mL in a TACE buffer described above, is mixed with a suitable crystallization buffer and crystallized using a suitable crystallization technique, for example drop vapor diffusion. Suitable crystallization buffers, for example, include: 0.1 M Na Acetate pH 5.3, 0.2 M $CaCl_2$, 30% v/v Ethanol; 0.1 M Na Citrate pH 5.0, 40% v/v Ethanol; 0.1 M Na Citrate pH 8.7, 20% w/v PEG 4000, 20% v/v Isopropanol; and 0.1 M Na Citrate pH 5.4, 20% w/v PEG 4000, 20% v/v Isopropanol. The sample is incubated at a temperature ranging from about 4 to 20 degrees Celsius. A crystalline precipitate is formed.

Seeds from the crystalline precipitate obtained, as whole crystals or crushed crystal suspensions, are transferred, along with a suitable crystallization promoter,

SPEC

Replace paragraph on page 18, line 17 as follows

such as hair of rabbit, to a solution of concentrated TACE/substrate in a crystallization buffer. Crystals suitable for X-ray data collection are formed.

Another aspect of the invention relates to a TACE polypeptide crystal. One such crystal comprises a TNF-α converting enzyme catalytic domain (TCD) polypeptide co-crystallized with an inhibitor. The crystal diffracts to about 2 A and belongs to the monoclinic space group $P2_1$. The crystal's unit cell comprises four crystallographically independent TCD molecules. The TCD molecules are in an asymmetric unit and are not clustered into separate tetrameres, but are integrated into the infinite periodic structure. The crystal has the cell constants: a=61.38 Å (angstrom), b=126.27 Å, C=81.27 Å and β=107.41°.

~~X-Ray Diffraction~~

Another aspect of the invention relates to the structure of TACE, particularly the structure of the TACE catalytic domain (TCD). The structure of TACE can be determined utilizing a crystal comprising a TACE polypeptide as described above. According to the present invention, the structure of TACE, and particularly the TCD, is determined using X-ray crystallography. Any suitable X-ray diffraction method for obtaining three-dimensional structural coordinates of a polypeptide may be used. The three-dimensional structure coordinates, or any part thereof that characterizes the part of the TACE polypeptide of interest, such as the TACE catalytic domain or part thereof that is capable of cleaving the peptide PLAQAVRSSS (**SEQ ID NO: 1**), can be used as described herein.

Methods of Using TACE X-Ray Diffraction Coordinates

The invention also relates to use of the structure coordinates obtained from the above described X-ray diffraction studies of the TACE catalytic domain. The coordinates may be utilized, by direct analysis, with the aide of computers, or combinations thereof, to determine the structure, including secondary and tertiary

SPEC

Replace paragraph on page 24, line 24 as follows

The C-terminal chain comprising the last 61 TCD residues (Fig. 3) first forms three short straight almost perpendicularly arranged segments linked by two "narrow" supertwisted loops, returns via the tight "Met-turn" Tyr433-Val434-Met435-Tyr436 back to the surface where it kinks at Pro437 to form the Pro437-Ile438- Ala439 outer "wall" of the S1' crevice, approaches in a wide loop the C-terminal α-helix hD and runs through it, and ends up on the molecular "back" surface close to the N-terminus, with the last defined residues Arg473-Ser474 fixed via hydrogen bonds to the main molecular body. Via Cys423-Cys453, the first of the two "narrow" loops is disulfide-linked with the N-terminus of helix hD, whose C-terminal end in turn is clamped to the "ear-like" sIV-sV linker peptide through Cys365-Cys469. Spatially adjacent, the third disulfide bridge of TCD, Cys225-Cys333, connects the N-terminal parts of β-strands sI and sIII. In the intact TACE molecule, four residues downstream of Ser474 would reside Cys478, which is already integral part of the compact elongated disintegrin domain (Saudek *et al.*, "Three-dimensional structure of echistatin, the smallest active RGD protein" *Biochem.* 30, 7369-7372 (1991)). Considering Ser474 and this Cys478 as pivot points of their respective domains, the three residue linker would allow relatively unconstrained docking of the disintegrin domain to the "left" surface side of the catalytic domain.

The active-site cleft of TACE (Fig. 2a) is relatively flat on the left hand (non-primed) side, but becomes notched towards the right. The catalytic zinc residing in its center is penta-coordinated by the three imidazole N∊2 atoms of His4O5, His4O9 and His415 (provided by the active-site helix and the following ''descending'' chain comprising the conserved zinc binding consensus motif HEXXHXXGXXH) (**SEQ ID NO: 3**), and by the carbonyl and the hydroxyl oxygen of the hydroxamic acid moiety of the inhibiter (see Figs. 1, 2a and 4). This zinc-imidazole ensemble is based on the distal ∊-methyl-sulphur moiety of the strictly conserved Met435, harbored in the Met-turn characteristic for the metzincin clan

(Bode *et al.*, "Astacins, serralysins, snake venom and matrix metalloproteinases exhibit identical zinc binding environments (HEXXHXXGXXH (**<u>SEQ ID NO: 3)</u>** and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'" *FEBS Lett.* 331, 134-140 (1993); Stöcker *et al.*, "The metzincins: Topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases" *Protein Sci.* 4, 823-840 (1995)). Both carboxylate oxygens of the "catalytic" Glu4O6 (which acts as a general base during catalysis (Grams *et al.*, "X-ray structures of human neutrophil collagenase complexed with peptide hydroxamate and peptide thiol inhibitors: Implications for substrate binding and rational drug design" *Eur. J. Biochem.* 228, 830-841 (1995)) squeezed between the zinc-liganding imidazole of His4O5 and the edge strand, are hydrogen bonded to the hydroxyl and the N-H group of the hydroxamic acid (see Fig.4). To the right of the catalytic zinc opens the deep S1' pocket, which, besides the S1' wall-forming segment (bottom, Figs. 1 and 2a), is bordered by the side chains of His4O5 and Glu4O6 (left), the sIV main chain and the Leu345 side chain (top), and the side chains of Val44O (back) and Ala439 (right). To the right of Ala439 opens a second (S3') pocket, which inside the molecule merges with the S1' pocket, leaving a small bridge made of the opposing side chains of Ala439 and Leu348 (Fig. 2a).

The (pseudo)peptidic part of the inhibitor binds in an extended geometry to the notched right-hand side of the active-site cleft, mimicking the interaction of the primed residues of a productively bound peptide substrate (Fig. 2a). It runs antiparallel to the upper short bulge Gly346-Thr347-Leu348 and parallel to the S1' wall-forming segment Pro437-Ile438-Ala439, making two and two inter-main chain hydrogen bonds, respectively. The dominant intermolecular interactions are made by the P1' isobutyl (pseudo-leucyl) side chain of the inhibitor and the essentially hydrophobic S1' pocket, however, is large and accommodates three partially ordered solvent molecules in addition. The P2' t-butyl side chain extends away

SPEC

Replace paragraph on page 27, line 12

associated C-terminal segments of three TNFα molecules (Jones *et al.*, "Structure of tumor necrosis factor" *Nature* 338, 225-228 (1989)) plays a role. In a productive TACE-proTNFα complex, the base of this TNFα-trimer cone (into which the disordered N-termini run up) may be recognized by the "right" side of the TACE catalytic domain (Fig. 2a), with the about 10 residues long spacer favoring the correct placement of the proTNFα Ala76-Val77 scissile peptide bond in the active site of TACE.

The polypeptide topology and in particular the surface presentation of the catalytic zinc prove the catalytic domain of TACE to be a typical metzincin. (Bode *et al.*, "Astacins, serralysins, snake venom and matmrix metalloproteinases exhibit identical zinc binding environments (HEXXHXXGXXH (**SEQ ID NO: 3**) and Met-turn) and topologies and should be grouped into a common family, the 'metzincins'" *FEBS lett.* 331, 134-140 (1993); Stöcker *et al.*, "The metzincins: Topological and sequential relations between the astacins, adamalysins, serralysins, and matrixins (collagenases) define a superfamily of zinc-peptidases" *Protein Sci.* 4, 823-840 (1995)) A superposition with the other metzincins shows, however, that its topology is most similar to that of the catalytic domain of snake venom metalloproteinases such as adamalysin II (Fig. 5). (Gomis-Rüth *et al.*, "First structure of a snake venom metalloproteinase: prototype for matrix metalloproteinases/collagenases" *EMBO J.* 12, 4151-4157 (1993); Zhang *et al.*, "Structural interaction of natural and synthetic inhibitors with the venom metalloproteinase, atrolysin C (form d)" *Proc. Natl. Acad. Sci. USA* 91, 8447-8451 (1994); Kumasaka *et al.*, "Crystal structure of H2-proteinase from the venom of Trimeresurus flavoviridis" *J. Biochem.* 119, 49-57 (1996)) This close homology is reflected by the much better simultaneous superposition of the central sheet and the large helices, but in particularly also by a couple of structural features, which TACE shares exclusively with the adamalysins such as: the long helix hB and the preceding multiple-turn loop arranged on top of the β-sheet; the typically arranged

SPEC

and shaped C-terminal helix hC; and the extended C-terminus placed on the backside surface. About 175 of the 263 TACE and 201 adamalysin α-atoms are topologically equivalent (with an rms deviation of 1.3 Å, 39 of which have identical side chains (Fig. 3). These numbers are close to those obtained from a comparison of members within the different metzincin families. (Stöcker *et al.*, *supra*) In addition, detailed structural features prove the close relationship of TACE to the adamalysins: a more conserved core structure; the loosely arranged N-terminus; the characteristic Asp416 (directly following the zinc binding consensus motif, Fig. 3) involved in identical intramolecular hydrogen bond interactions; the adjacent disulfide bridge Cys423-Cys453 linking the first narrow loop to the C-terminal helix hD (which TACE does not share with adamalysin II, but with the H2-proteinase from the snake venom of T. flavoviridis) (Kumasaka *et al.*, *supra*); disulfide bridge Cys365-Cys469 connecting the sIV-sV linker with the C-terminal helix hD; a similarly shaped active-site cleft, with particularly strong similarities in the SI' pocket and other primed subsites.

The catalytic domain of TACE (TCD) also differs from adamalysin II in several respects: with 263 residues, its chain is much longer; most of the additional residues of TACE are clustered giving rise to a more projecting hA-sII turn, to the two surface protuberances of the multiple-turn loop, to the two "ears" of the sIV-sV linker, and to a more bulged-out sV-hC connector (see Figs. 3 and 5); lack of a calcium binding site but presence of a third disulfide bridge Cys225-Cys333 in TACE, both elements serving, however, for the same function namely to clamp the N- terminal chain to strand sill; the quite deep S3' pocket of TACE which merges with its S1' pocket; an almost inverted charge pattern in and around the primed subsites, with an absolute predominance of positive charges in adamalysin.

According to its sequence, and probably with respect to its three-dimensional structure, the TACE catalytic domain is thus not a typical member of the mammalian ADAMs proper (a family of membrane-anchored cell-surface proteins,

SPEC

Replace paragraph on page 36, line 8 as follows

~~Attorney Docket No.: 16761/1~~

~~Example 2 - Protein Crystallization~~

A DNA construct comprising the prodomain and the catalytic domain of human TACE (resides 1-477) was fused to the sequence Gly-Ser-$(His)_6$ **(SEQ ID NO: 2)** to facilitate purification of the protein on a Ni-NTA affinity column. Chinese Hamster Ovary (CHO) were cells used for protein expression. The cells secreted a mixture of mature TACE beginning with either Val212 or Arg215. TACE-containing fractions from the Ni-NTA column were incubated in a buffer containing octylglucoside and the binding partner N-[D,L-[2-(hydroxyaminocarnbonyl)methyl]-4-methyl-pentanoyl)-L-3-(tert-butyl)-glycyl-L-alanine. The final purification step was performed on a gel filtration column. Purified TACE was stored in a buffer containing 10 mM Tris/HCL pH 7.5, 100 mM NaCl, 10% glycerol and 1 mM of inhibitor (TACE buffer).

~~Crystallization experiments were set up at a TACE concentration of approximately 5 mg/mL by mixing TACE (in TACE buffer) in a 1:1 ratio with the crystallization buffers listed below and using the sitting drop vapor diffusion technique. The experiments were performed in duplicate and incubated either at about 4°C or at 20°C. Crystalline precipitate was obtained at 20°C in the following crystallization buffers:~~

~~Buffer A) 0.1 M Na Acetate pH 5.3, 0.2 M $CaCl_2$, 30% v/v Ethanol~~

~~Buffer B) 0.1 M Na Citrate pH 5.0, 40% v/v Ethanol~~

~~Buffer C) 0.1 M Na Citrate pH 8.7, 20% w/v PEG 4000, 20% v/v Isopropanol~~

~~Small crystals were obtained upon transferring seeds from the crystalline precipitate with a hair of a rabbit into a 1:1 mixture of a concentrated sample of TACE (12 mg/mL in TACE buffer) with either buffer B or C. Further refinement of buffer C resulted in buffer D, which allowed the production of crystals suitable for X-ray data collection.~~

~~Buffer D) 0.1 M Na Citrate pH 5.4, 20% w/v PEG 4000, 20% v/v Isopropanol~~